GROWTH AND DIFFERENTIATION OF DAPHNIA MAGNA EGGS IN VITRO

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It is the purpose of this paper to give an account of the development of *Daphnia magna* eggs *in vitro*. To our knowledge the method presented here of growing parthenogenetic eggs of *Cladocera in vitro* instead of in the brood-chamber of the mother represents the first endeavor in this direction. The study was undertaken because in the course of some ultra-centrifuging experiments two years earlier there arose the need of rearing the eggs of this animal in tissue culture slides. The photomicrographs and the brief description of the developmental stages in the course of the growth of the organism outside the brood-chamber of the mother are intended at this time to serve only as a basis for a more critical attack on a large number of possible problems with *Cladocera* in general.

Methods

The animals employed in this work can be traced to Anderson's stock (1932) and this in turn to animals received from Banta's laboratory. This particular species was utilized previously in our laboratory for a period of about five years for other experimental work. The animals were reared only parthenogenetically and they represented genotypically identical individuals derived originally from a single animal.

A number of different media were tried in an endeavor to grow eggs of *Daphnia magna* successfully *in vitro*. A modified Ringer's solution, utilized by Lévy (1927) for physiological work on isolated organs of *Cladocera*, injured the eggs, and in no case was complete development effected. Likewise an endeavor was made to imitate the cultural condition of the brood-chamber by the introduction of varied amounts of the fluid obtained from the brood-chambers of animals in which there were developing eggs. The growth of eggs in such a medium did not show any advantage over a simpler medium consisting of sterile pond water. This is of considerable interest in view of the nutritive rôle which has been ascribed to the fluid present in the brood-chamber (Dearborn, 1908; Birge, 1918). Contrary to expectation, carefully controlled experiments with eggs reared *in vitro* in a medium consisting of sterile pond water repeatedly showed 100 per cent egg development. Such eggs gave rise to fully developed young in about the same period of time as the eggs developing in the brood-chamber of the mother. These observations establish without any doubt the nutritive sufficiency of materials already stored in each egg at the time of laying, and they also throw some light on the nature and the possible function of the tough membrane which surrounds the developing egg.

A *Daphnia magna* female, the eggs of which were to be removed from the brood-chamber, was allowed to swim for a few minutes in a vessel of pond water which had been rendered sterile by heating (distilled water appears to be injurious to the organism). This procedure freed the animal to some extent from microorganisms which were found to cling to the body and which, when present in the egg medium, interfered with the normal growth of the embryos. The animal was then placed in a depression slide (the type commonly employed for tissue cultures) with a round polished cavity 15 mm. in diameter and 3 mm. deep and the slide was filled with sterile pond water. With the aid of a fine dissecting needle, which was placed over its head, the animal was held in the field of the binocular microscope. Another dissecting needle was applied to the inside of the lower carapace, and the animal was held in place on its side against the surface of the slide. The needle which held the head in place was then transferred to the inside of the upper carapace. The two flaps of the carapace were then spread apart till the upper flap was made to lie against the slide. This procedure exposed the eggs in the brood-chamber. By gently moving the body of the animal back and forth with the dissecting needles, while the flaps of the carapace were still held against the slide, the eggs were made to roll out of the chamber without being subjected to any pressure in the course of the dissection. The remains of the animal were then removed and the water surrounding the eggs was replaced by fresh sterile pond water. The successful removal of eggs from the brood-chamber of the mother without any injury to them determines to a large extent the degree of success in the rearing of the eggs of this animal *in vitro*.

To determine the time required for eggs to complete their development in the brood-chambers of the animals, individual animals were isolated in bottles containing the standard amount of the culture medium (Banta, 1921). On each bottle was recorded the exact time when the eggs were deposited. The animals were reared at 25° C. in a water bath, the temperature of which was controlled to ± 0.01° C. From repeated observations on animals reared under such conditions, it was found that with very few exceptions mothers released their young some time during the forty-sixth hour after the deposition of the eggs.

Many series of developing eggs *in vitro* were carried to completion. The length measurements referred to in this paper and the degree of development of the embryos illustrated in the photomicrographs represent observations on a single clutch consisting of 22 eggs. For other observations made simultaneously with this clutch of eggs, there were carried other parallel series *in vitro* and also eggs developing in the brood-chambers of mothers, serving as controls. In an endeavor to perfect the technic of recording the morphological changes in the course of the development, at least 4 other series of photomicrographs were completed and each one of these was accompanied by other parallel series of eggs growing in depression slides and also developing in the brood-chambers of mothers. *Daphnia magna* eggs when reared *in vitro* were found likewise to complete their embryonic development in about 46 hours at 25° C. It was observed, however, that if the growing eggs were subjected to a lower temperature in the course of routine examination or in the course of replenishing from time to time the fluid in the culture slides, there was a delay of several hours in completing the embryonic development. It was found that whenever a young in the tissue culture slide corresponded in development to one just released from the brood-chamber, it extended its caudal spine which up to this time had been closely adhering to the post abdomen (Fig. 15) and immediately began to exhibit more active swimming movements. The extension of the caudal spine and the more active swimming movements which followed were utilized by us as criteria in judging the end point of embryonic development *in vitro*.

Gross Microscopical Examination of Developing Eggs in Vitro

Inasmuch as the embryos are transparent, the rearing of *Daphnia magna* eggs on tissue culture slides enabled us to make direct observations on some of the sequence of events in the course of development of the embryos without the necessity of staining and sectioning.

The parthenogenetic egg of *Daphnia magna* is enclosed in two membranes. The inner membrane is the vitelline and closely adhering to it on the outside, observable even in eggs a few minutes after their deposition, there is a more prominent membrane. Lebedinsky (1891), in a short account of the development of *Daphnia similis,* refers to this membrane as the chorion. These two membranes exhibit a progressive resistance to mechanical injury with lapse of time from the deposition of the eggs. This was repeatedly demonstrated by subjecting the eggs to ultra-centrifuging at various stages in their development. A comparatively weak centrifugal force consisting of 1800 times gravity was sufficient to rupture the membranes and bring about a disintegration of newly deposited eggs the moment the ultra-centrifuge was started. Eggs three hours after their deposition withstood the same centrifugal force for several minutes without disintegration. Still older eggs were subjected to a centrifugal force of as much as 50,000 times gravity for several minutes without a bursting of the membrane. In our study three-hour-old eggs were usually chosen for growth in tissue culture slides. Such eggs invariably attained 100 per cent development. Earlier eggs may be reared with equal success, but greater precautionary methods must be employed during the dissection of the mother and in the subsequent handling of the eggs.

The first morphological change noticed in the course of development *in vitro* is the appearance of an invagination in eggs nine hours old, reared at 25° C. This invagination gives the first indication of the prospective cephalic region (Fig. 3). It becomes more prominent in the next three hours of development (Fig. 4). At this stage a vertical constriction appears also at the extreme posterior end (lower edge in the figure), which marks the bilateral symmetrical plan of organization which is to follow.

During the first 18 hours of development the eggs remain nearly perfectly spherical and do not show any increase in size. The clutch of eggs studied under very careful control, measured throughout this period 0.275 mm. in diameter. Figure 5 shows an egg 18 hours old. This egg, although it is of the same size as in the previous stages, shows the beginning of the cephalic appendages and also some slight evidence of the beginning of the abdominal appendages.

The prospective left and right halves of the future organism are well marked in embryos 21 hours old (Fig. 6). At this stage the embryos measured 0.337 mm. in length in contrast to 0.275 mm. which characterized the length of all the previous stages up to this point. This represents an initial increase in length of about 60 μ. At this same stage there appears also mid-dorsally at the cephalic region a blastodermic thickening, which constitutes the first external evidence of brain development. The prospective cells which are to form the brain are definitely recognizable earlier in prepared slides. In eggs 24 hours old (Fig. 7) this organ is unmistakable when viewed grossly under the microscope.

In embryos 27 hours old (Fig. 8) there comes into prominence, dorsal to the prospective brain, a mass of granular substance at first grayish in appearance, then, with subsequent development becoming brown and finally black. This granular material, which at first is represented by a single mass, in the 30-hour embryo, is seen to differentiate into two well-established masses now brownish in color, representing the material for the development of the eye (not in focus in the photomicrograph, Fig. 9). This double mass is clearly shown in Fig. 10, representing an embryo 33 hours old. Anterior to the eye masses there is visible also the dense material for the prospective ocellus. The eye in a fully developed individual is represented by a single organ placed in the front of the head, composed of ommatidia. It is interesting to note that this cyclopian eye of *Daphnia magna* has embryologically a double origin. Whether or not the sequence of events involved in the development of this organ has phylogenetic significance remains to be investigated. So gradual and orderly is the fusing of the eye masses into a single structure that we are utilizing this process in our laboratory as a criterion in judging the stage of the development of the embryo *in vitro,* also in predicting the time of the release of the young from the brood-chamber.

The first sign of movement of the body was observed in embryos 30 hours old. This was characterized by slow lateral expansions and contractions of the entire body, resembling massaging motions. There were also observed in the embryo at this stage a few irregular heart

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PLATE I

Photomicrographs of *Daphnia magna* eggs and embryos in the course of their development *in vitro*. Figures 1, 2, 3, 4 represent prints from negatives. Figures 5–12 represent prints made by direct projection of the living material on Defender Velour Black paper utilizing a Bausch and Lomb vertical microscopical slide projector. They are negatives. The magnification is the same throughout. × 117.

FIG. 1. Egg 3 hours after deposition.

FIG. 2. Egg 6 hours old showing the beginning of the formation of a clear zone around the periphery.

FIG. 3. Egg 9 hours old showing the first invagination. The interior light areas in Figs. 1–3 are oil droplets.

FIG. 4. Egg 12 hours old showing the chorion and the first demarcation of the future cephalic portion. A constriction at the extreme posterior end marks the beginning of the bilateral symmetrical plan of development.

FIG. 5. Egg 18 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIG. 6. Embryo 21 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIGS. 7, 8, 9. Embryos 24, 27 and 30 hours old respectively, showing further developmental differentiations.

FIG. 10. Embryo 33 hours old, showing the early appearance of the two eye pigment bodies and posterior to them the ocellus, the black bodies surrounded by a clear area, centrally placed in the cephalic region. These bodies are not in focus in the earlier stages.

FIG. 11. Embryo 36 hours old, showing the further differentiation of the two eye masses and the ocellus, the white bodies at the extreme anterior end.

FIG. 12. Side view of an embryo 39 hours old after infolding of the cephalothorax.

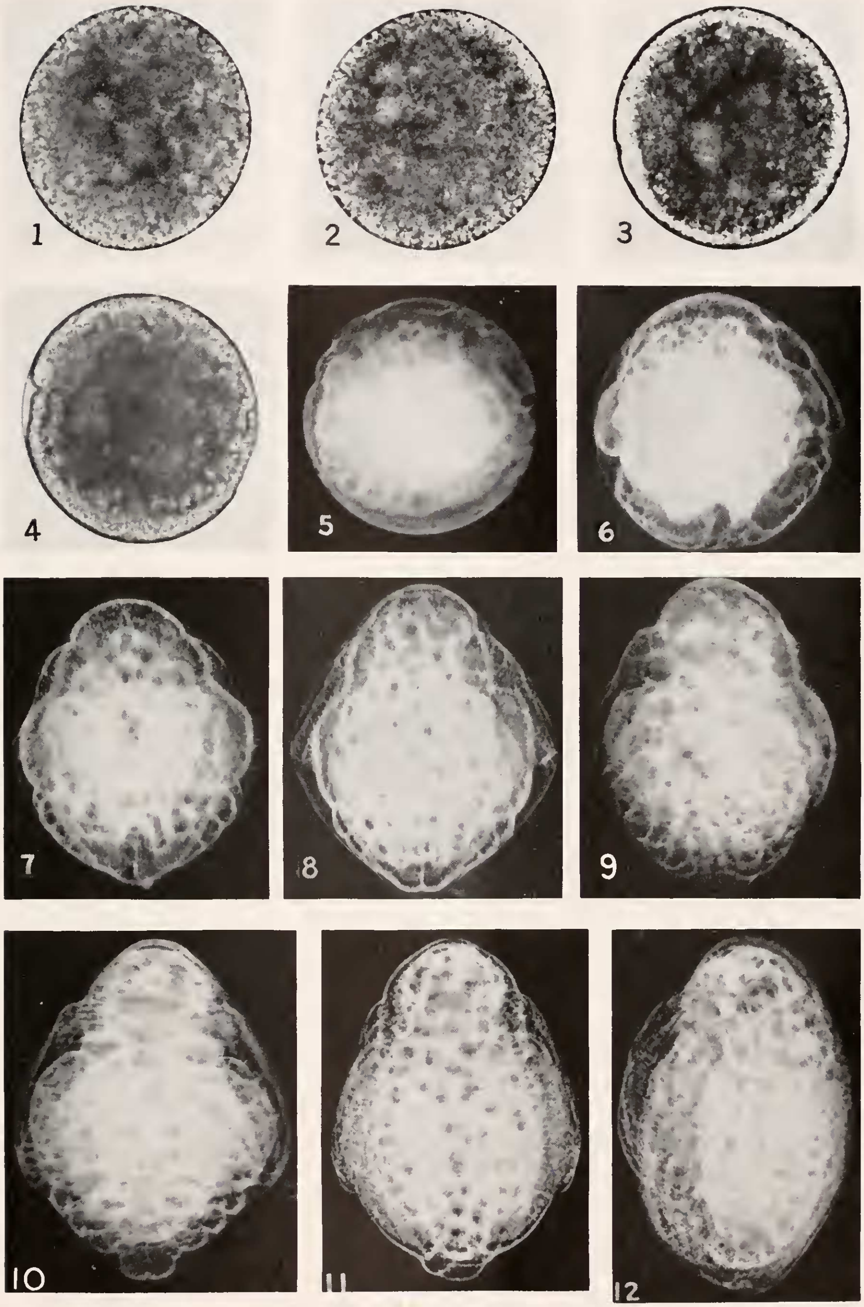

PLATE I

beats per minute. Five hours later, the heart beats increased to about 20 per minute and in 36-hour embryos the rate became 32 per minute. With the further differentiation of the organism, it became more regular and more rapid. Fully formed young when in their second instar show a heart-beat rate of about 180–200 per minute but this varies greatly with the temperature. The observations recorded here were all made at 19° C. ± 1° C. It is evident, therefore, that the embryonic rate of the heart beat of this organism is less than that in the adult.

The increase in body length in the course of the embryonic development of *Daphnia magna in vitro* is not continuous. There are periods during which no increase in length can be observed, during which time undoubtedly other changes are taking place. As already pointed out, the first increase in body length of about 60 μ was seen in embryos 21 hours old. In the next 3 hours of development there was an additional increase of about 40 μ, and in embryos 27 hours old the body length became 0.41 mm., representing a further growth of about 30 μ over the previous stage. After this there followed a 9-hour period during which the embryos increased in width rather than in length (30, 33 and 36-hour stages). This is more clearly demonstrated in Fig. 10 showing an embryo 33 hours old, just before the infolding of the cephalothorax has begun. After the 36-hour stage and for the next 9 hours there was again observed a gradual increase in body length varying from 20 μ to 60 μ for each 3-hour period of development. The most pronounced increase in body length took place during the hour just previous to the completion of the embryonic development. It appears that with the extension of the caudal spine (Fig. 14) there is also freed a portion of the posterior carapace heretofore held in place. This may account for the apparent sudden increase in the length of the embryo of about 180 μ in one hour (excluding the caudal spine) which is considerable considering the previous increments of growth. This sudden increase in growth may be also due to imbibition of water at this period, a phenomenon so characteristic in fully grown individuals between their successive instars. In some *Daphnia* Miss Rae Whitney of Brown Uni-

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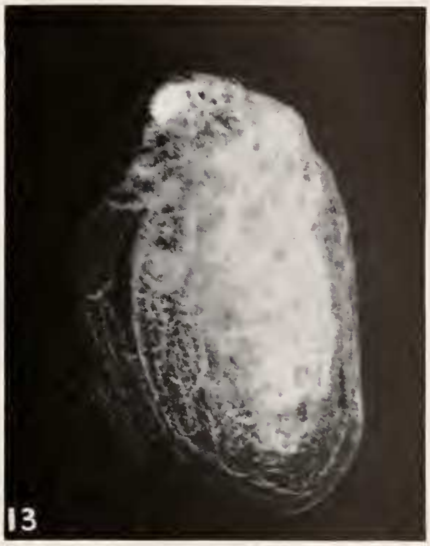
PLATE II

Photomicrographs of *Daphnia magna* embryos in the course of their development *in vitro*, made by direct projection of the embryos on Defender Velour Black paper. They are negatives. The magnification is the same throughout. × 130.

FIG. 13. Embryo 42 hours old showing the eyes laterally placed, and the caudal spine closely adhering to the post-abdomen.

FIG. 14. Embryo 45 hours old—one hour before it emerges as a fully developed young. The caudal spine is only partially extended.

FIG. 15. Embryo 46 hours old representing a fully developed young. The caudal spine is now fully extended.



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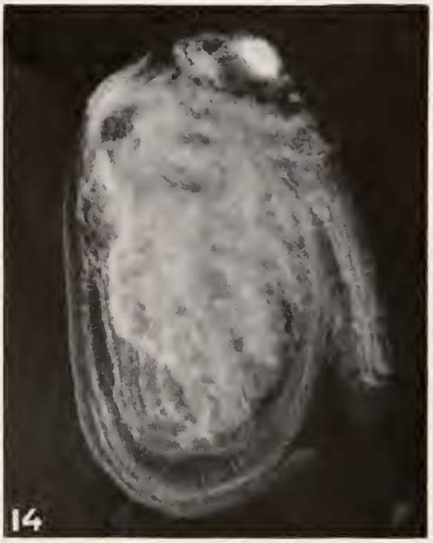

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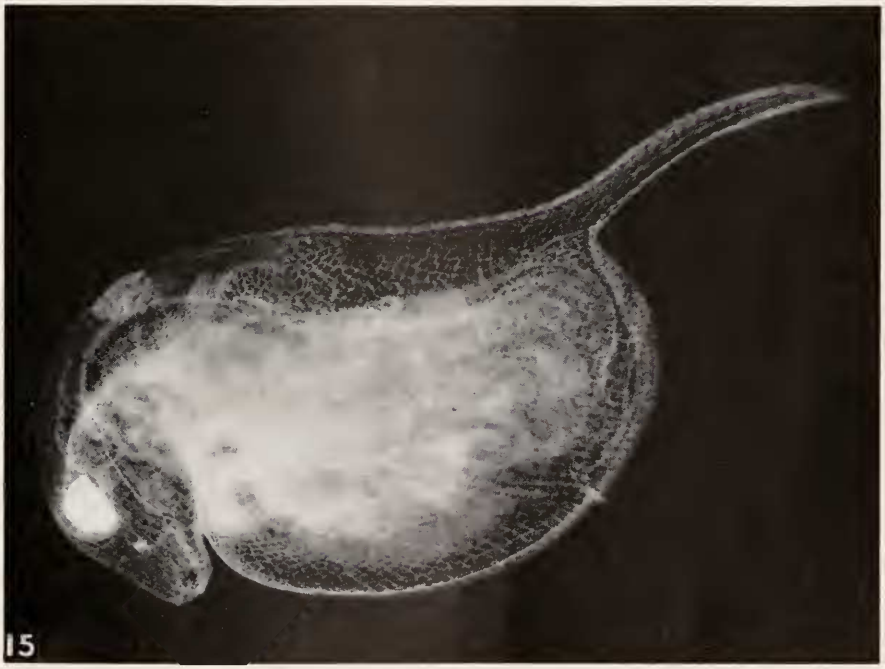

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PLATE II

versity finds a pre-natal molt at this stage of the development (unpublished observations).

After the extension of the caudal spine (Fig. 15) the individuals resemble in every respect young just freed from the brood-chambers of mothers. They immediately become more active, utilizing to a fuller extent in their locomotion the antennae and other appendages, thus completing a history of embryonic development from eggs to independent free-swimming organisms in about 46 hours. The individuals at this stage measure about 0.74 mm. in body length. When such individuals are transferred to bottles containing a standard amount of culture medium (Banta, 1921), but at first in a more diluted form, they, too, become mature and in time produce other young.

Summary

1. The parthenogenetic eggs of *Daphnia magna* may be reared in sterile pond water on tissue culture slides, completing their embryonic history from egg to free-swimming independent organisms in about 46 hours.

2. The eggs of this animal are self-sufficient with regard to the nutritive materials already stored in them at the time of their deposition. Such eggs are transformed into fully developed young *in vitro* in about the same period of time as that required for the development of eggs in the brood-chamber of the mother.

3. Fully developed young reared *in vitro,* when transferred to bottles containing the standard amount of culture medium, become in time sexually mature and produce young.

4. A brief description is given of the sequence of events in the course of the development of the embryos reared on tissue culture slides, as observed by gross microscopical examination.

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